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The Development of a Method For the Separation of Diastase From Organic Materials And from Catalase

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THE DEVELOPMENT OF A METHOD FOR THE SEPARATION OF DIASTASE FROM ORGANIC MATERIALS AND FROM CATALASE

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CONTENTS.

THE DEVELOPMENT OF A METHOD FOR THE SEPARATION
OF DIASTASE FROM ORGANIC MATERIALS
NOT FROM CATALASE.

1. Introduction.

Most of the experimental work with enzymes and other similarly obscurely known substances e.g. the immune bodies, is conducted with preparations which contain the desired body in admixture with other, in most cases quite undesirable, substances. The nature and condition of the extraneous matter is very obscurely known.

It is furthermore not possible in the use of such preparations to reproduce exactly the same conditions in the repetition of experiments. The validity and range of application of the results obtained then remains unknown. It is largely for these reasons that the literature on the biology and chemistry of the enzymes, immune and similar bodies exhibits so many directly contradictory reports. It is evident that any experimental results however small that contribute toward the separation and identification of these substances are worthy of the necessary effort. It is also clear that progress of a fundamental nature will be possible only as advance has been made in this direction. For these reasons the present work on diastase was directed toward this vital point. Our purpose must not be misunderstood to have been the production of the imaginary chemically pure diastase. Our present ideas about such an imaginary substance may be irrational and we have not even a certain conception of what properties this supposed body should exhibit. Our aim has confined itself to the practically attainable end, as the result of these experiments show, of preparing a diastase in fairly pure and practically constant con-

dition, with which experiments can be definitely repeated, and which may serve as the point of departure for the ultimate preparation of pure diastase if that should be feasible. In short we have developed several methods of adsorption, to one of which, that of adsorbing diastase from an aqueous or acetone solution with normal lead phosphate, we feel justified in giving the confidence resulting from considerable experimental testing. These tests are not yet completed but the results thus far attained are sufficient to place the method upon an assured basis of fact. We have not had time to make more than a beginning as shown in this paper, in answering interesting questions that become approachable after one preliminary separation of the enzyme in definite condition. In making some of these latter experiments the advantages for some purposes became evident of dealing with the diastase in practically solid conditions, which permits the action and removal of various agents and subsequent examination of the results produced. It ought to be noted here that the adsorption of several enzymes by the same adsorbent constitutes not so much a source of difficulty as of theoretical and practical considerations of wide range. This aspect, also, of the method has not yet received full investigation. It should not be inferred from the above description that the use of the ferment in adsorbed condition is a necessity of our method as a reference to the description of the experiments will show that the ferment can, to a considerable extent be dissolved from the adsorbent in very good condition. The adsorption of other ferments which was studied by other experimenters in parallel with that here described will be reported in separate papers.

2.1 Toluol-Thymol Antiseptic.

In the following experiments where an antiseptic was necessary a mixture of 100. cc. toluol containing about 0.5 gm. thymol was used. In most cases a 50.% acetone solution was used as a solvent for the diastase. Where such a strong solution of acetone remained present without dilution no additional antiseptic was necessary for experiments of short duration.

2.4 Estimation of Sugar by Schoorl's Modification of Fehling's Method.

I. 8. cc. of strong Fehling's solution.

II.(a)Add H_2O for the blank test up to 20.cc.i.e.dilute 2½ times.

(b) Add solution to be tested to the 8. cc. of strong Fehling's solution until a volume of 20. cc. is reached, i.e. dilute 2½ times.

III. (a) Rapidly bring the solution to the boiling point and boil for 2 minutes, then cool under tap at once, and reduce to room temperature.

IV Add 8. cc. of a 10.% solution of KI also add 4. cc. of a 25.% solution H_2SO_4.

V. Add a small amount of starch in taking the blank test. This is omitted in the regular determination. Now titrate the solution with $Na_2S_2O_3$. Add 0.1 N. at a time until a creamy white milk color is reached. This is the end point.

VI. Calculation- The amount of $Na_2S_2O_3$ used in the blank minus the amount used on the solution tested equals the amount of CuO reduced. Find this amount on Schoorl's table, by the number of cc used and reduce the same to milligrams, and the percent equals the amount of sugar reduced.

.2 Separation of Diastase and Catalase.
Experiment Number 8-4-7/1.

The object of this experiment is to show that a 50% acetone solution does not extract catalase, neither does it denature it under the conditions considered.

Malted wheat, as explained below, was treated with a 50% acetone solution and allowed to extract from one to five days. This extract was designated as number 8-4-2. The liquid of this extract was tested for catalase with hydrogen peroxide and there was none found. The wheat particles were then removed from the extracting mixture and washed with distilled water, and also tested. In this case a good test for catalase was obtained.

According to this experiment the catalase was present in the solid material, but the 50% acetone solution failed to extract it.

Experiment Number 8-4-7/1.

The wheat extract used in the experiment 8-4-7/1 is here tested for diastase. 25.0c. of the original acetone extract of wheat, number 8-4-2, was filtered through a cellulose-aluminum phosphate prepared filter, whose preparation is described below, and afterwards washed with distilled water. This removed the adhering sugar as was determined by Fehling's test. A portion of the filter, including paper and aluminum phosphate, was incubated with a 1% starch solution, whose preparation will also be subsequently described. After incubating 1 hour at 50°C. sugar was obtained by Fehling's method. No sugar was obtained at the beginning of the period of incubation. The remaining portion of the prepared filter, containing the diastase was tested directly for catalase with hydrogen peroxide, but there was none found.

Experiment Number 8-4-31/1.

The object of this experiment is to determine whether the prepared filter gives any reduction of itself.

The aluminum-phosphate filter was prepared as in previous experiment number 8-4-7/2 and incubated with an equal volume of a 1.% starch solution for one hour at 37°C. A test for sugar was then made by Fehling's method, the result being negative.

Experiment Number 8-3-16/2.

This experiment was performed to determine the effect of acetone upon Fehling's solution in the preserse of starch. One volume of a 1.% starch solution and one volume of concentrated acetone was boiled with two volumes of dilute Fehling's solution. There was no reduction.

The above experiments show that the diastase may be extracted from wheat with a 50.% acetone solution, and that the catalase remains undenatured in the solid material.

2.3 Preparation of Starch Solution.

About 75. cc. of distilled water was heated to boiling in an evaporating dish, and allowed to cool to about 60°C. One grm. of Kohlbaum's soluble starch was now added with agitation until thoroughly dissolved. When the solution was cool the entire amount was made up to 100. cc. with distilled water. A fresh solution of this kind was made each day.

3. The Production and Extraction of Diastase of Different Origin.

So far as known all plant and animal tissues contain a diastatic enzyme. Representative conditions will be considered and extraction methods applied to each.

31 Composition and Preparation of Bacterial Culture Liquid.

The culture liquid in which the bacteria were grown consisted of :- 1 Gram of Kahlbaum's soluble starch; 1 gram of Witte's peptone; 1 gram of Merck's potassium phosphate, KH_2PO_4, and one drop of U. S. P. tincture of iron, all made up to 1000 c.c. with distilled water. It is best to dissolve the starch and peptone in hot distilled water and when cooled add the other ingredients.

The above culture liquid was seeded with about 10. c.c. of original material, obtained as explained below, and placed in the incubator at 37.° C. for ten to twelve hours. Under such condition the bacteria multiply very rapidly. After this preliminary incubation the bacterial cultures are kept at room temperature.

Method of Obtaining and Cultivating Strains of Diastatic Bacteria.

The original material for the bacterial culture liquid consisted of water, carrying in suspension both decaying vegetable matter and some of the sediment, which is found in a layer at the bottom of a polluted stream. This liquid was obtained from the Bone Yard, a small sluggish stream at Urbana, Illinois, and taken immediately

to the laboratory where it was thoroughly mixed. A small portion of this, about 10. c.c. is the original material with which the first of these bacterial cultures was seeded. New culture liquids were made, as described above, about every week, and seeded from the one previously made. In this way diastatic bacteria were kept in the laboratory for experiment.

Data on Bacterial Culture.
(Experiment number 8-3-30.)

Date	Iodine test for starch	Dil. Feh. test for sugar	Biuret test for protein	Time of incubation at 50.° c.	Reduction of Wehling.
8-3-30	Strong test	None			None
8-3-31	None	"	Violet	2 hours	Very slight
8-4-1	"	"	Violet to red	"	Increased amount
8-4-2	"	"	Slight test	"	Good reduction
8-4-3	"	"	Very doubtful	2-45	"
8-4-4	"	"	None	3hours	"

Explanation of Data on
Experiment Number 8-3-10.

The above results were taken each day at practically 24 hour intervals. We find that no trace of starch was obtained after the first day. At no time was sugar present in sufficient quantities to be detected by the Fehling test. The protein compounds gradually changed as indicated by the various tests from violet, violet red, to reddish tinge, and finally no reaction was obtained. A fermentation test for the developement of diastatic power by the bacteria was made daily. The temperature and time of incubation were practically uniform. Judging from the reduction of Fehling's solution, as described in the last column of the preceding table, there was a marked developement of diastatic power during the first few days. After this developement the bacteria were found to continue in diastatic condition indefinitely for a week or longer. The bacteria were always killed, as will be described below, previous to the incubation test.

Extraction with 50.% Aqueous Acetone.

It has been repeatedly found that diastase is not precipitated by 50.% aqueous acetone in any materials I have examined. After the initial action of bacteria there are practically no precipitable proteins remaining. The starch also was quickly decomposed. The experiment showed that the addition of an equal volume of concentrated acetone to a portion of the bacterial culture liquid produced no precipitate. Hence in liquids which have this perfect degree of decomposition, the acetone treatment is omitted. This thorough decomposition due to bacterial metabolism stands in strong contrast to the liquids obtained from autolytic liver, or germinat-

ing heat, which always gave an abundant precipitate with acetone. Of course in these last instances the action of bacteria is excluded by toluol-thymol.

32 Autolytic Extract of Liver.

The liver used in preparing the extracts was obtained at the meat market. It was the same as that which is sold for food. The tissue was cut into small bits, and placed in distilled water of about three times the weight of the tissue used. The tissue was thoroughly agitated with toluol-thymol and set away to digest in a glass jar.

By this method the maximum amount of diastase would occur in about a week after setting. After that time the solution seemed gradually to loose its diastatic power.

Separation of Diastase with 50.% Aqueous Acetone Solution.
Experiment Number 8-4-8/2.

This experiment was performed to decide whether diastase could be extracted from meat by means of a 50.% acetone solution.

16. Grams of steak were treated with 32. c.c. of a 50.% aqueous acetone solution, at room temperature for 24 hours. At the end of this time a portion of liquid was withdrawn and centrifuged. 10. C.c. of the clear liquid was incubated with an equal volume of a 1.% starch solution at 40°C. Before incubating however a Fehling's test for sugar was made, with a negative result. After incubating for 2 hours Fehling's test for sugar was again made, and a good reduction was obtained.

Control Experiment on 8-4-8/2.

This experiment was to determine whether the reduction obtained in 8-4-8/2 was from the presence of the acetone.

Equal volumes of a 1.% starch solution and a 50.% aqueous acetone solution were incubated for 2 hours at 40.°C. After which a Fehling's test for sugar was made, with a negative result.

These experiments show that a 50.% acetone solution will extract diastase from meat.

37 Malting Wheat with Thymol as Antiseptic.

Extraction of Diastase with 50.% Acetone.

Ordinary wheat was thoroughly washed in tap-water and allowed to soak for about twelve hours. At the end of this time the water was drained off, and the swollen grains were placed in a copper germinating pan upon a moist filter paper. Thymol water was now sprinkled over the wheat grains and a cover placed on the pan. The pan was kept in a warm room and allowed to remain three or four days, or until the wheat had germinated. The tops of the pans were now removed and the wheat allowed to dry at room temperature. After drying the wheat was ground in an ordinary coffee mill and treated with a 50.% aqueous acetone solution to extract the diastase. The proportions used were 1 gram of ground wheat to 10.c.c. of 50.% acetone. Room temperature was used for this extraction.

4. Separation of Diastase from Autolytic Products by the Acetone-Tannic Acid method.

Diastase as found in plant and animal tissue may be separated from autolytic decomposition products, by what I wish to call the Acetone-Tannic Acid method. This method has general application, with only slight modifications, and I shall now present results from representative raw materials. The various steps of the process are designated by A. B. C. etc.

In the application of the Acetone-Tannic Acid method to liquid bacterial cultures, the following reagents were used. (1) Albumin; which is prepared as follows;- The albumen from a hen's egg, free from yolk is treated with 10 volumes of distilled water, which dissolves the albumin, and precipitates the globulin, which latter may be removed by filtration through cotton. A few drops of benzol is added to the dilute albumin solution as a preservative..(2) A 50% solution of Kahlbaum's tannic acid was used, which does not reduce Fehling's solution. To this was added a few drops of acetic acid.

Process A.

The bacterial liquid culture was well shaken with some toluol-thymol and let stand for some time to kill the bacteria. The method of production of the bacterial culture is given above. The toluol-thymol being lighter than the culture liquid soon rises to the top, and portions of the liquid from underneath are easily withdrawn by means of a pipette.

Process B.

The bacterial liquid culture is treated with an equal volume of tannic acid reagent, described above, and also about 1/3 volume of albumin reagent. It is allowed to stand until precipitation is complete, and it is then passed through a Schleicher and Schüll's number 595 wet filter paper. The precipitate is now washed with distilled water on the filter paper until the adhering sugar, and the excess of tannic acid are removed. This is determined by the Fehling's and iron tests respectively upon the wash water.

Process C.

The filter paper is now removed and immediately placed in a flask containing a 50.% acetone solution, and shaken until the filter paper is disintegrated and the precipitate is finely divided. This should now stand for an hour or two to extract; then the solution is filtered through a Schleicher and Schüll's number 595 filter paper as above described. The filtrate thus obtained contains a very active diastase and is practically free from both the protein and the dissolved extraneous substances of the original material. The diastase solution may be used at this stage, but a purer product may be obtained, as subsequently described in the processes D. and E. A discussion of steps A. B. and C. will also be given below.

The process A depends primarily upon the fact that the ferment diastase whatever its chemical nature may be is readily soluble in 50.% aqueous acetone.

It is true, of course, that concentrated acetone might precipitate it, and the use of 50.% acetone is the result of experience.

Another highly important property of the acetone solvent is its ability to precipitate, or to fail to dissolve, practically all proteins, excepting those which are alcohol soluble. Other colloids such as starch glycogen, dextrine, etc., are also for the most part kept out of the solution by this concentration of acetone. Hence the process A furnishes a liquid approximately free of colloidal protein and carbohydrates, but containing, it must be noted all the crystalline decomposition products of an autolytic or extraneous character. By the use of this solvent a very considerable removal of undesirable substances from the diastase is accomplished.

In the process B there is obtained by precipitation with tannic acid the diastase and possibly some of the colloids of protein or carbohydrate nature which may have dissolved into the liquid A.

But the soluble and crystal decomposition products remain mostly in solution, and by the rejection of the filtrate from B, most of the soluble non-diastatic substances are removed from the diastase. By a minimum solution of adventitions substances and the rejection of those which are soluble there is accomplished an effective step in freeing the diastase from other substances. We have now to proceed with a precipitate containing the diastase and other colloids.

Fortunately for our purpose, 50% acetone has been found a good solvent for diastase, even after it has been precipitated with tannic acid. The other colloids seem to be insoluble in this reagent excepting, of course, the alcohol soluble proteins. Some acetone alcohol soluble protein seems always to have been present. By

filtering and washing, the precipitate is freed from the excess of tannic acid, and then by digesting the precipitate for about 24 hours in 50.% acetone there is obtained an approximately clean solution of diastase. This liquid usually gives the tannic acid reaction with iron. The whole process A. B. C. must be regarded as an approximate, but fairly good, method of obtaining a diastase which is much better than the original condition of admixture with numerous undeterminable substances. As will be described subsequently, it was found that a much superior method depending upon adsorption could be applied to the product C, or even to A.

Control tests on the various steps in the separation of diastase from bacterial liquid culture are as follows:- A. The bacterial liquid culture, as prepared above, was found to be diastatically active after two hours from time of setting, which is an exceptionally short time . A portion of the bacterial liquid culture was treated with toluol-thymol to kill the bacteria, and after a few minutes was incubated

with an equal volume of a 1.% starch solution at a temperature of 50°C. A sugar test was made by the Fehling method before setting however, and no reduction resulted. After incubating one hour a good reduction was obtained by Fehling's test for sugar. Control tests were made upon the starch and Fehling's solutions used, by incubating the starch, using toluol-thymol as antiseptic, and testing the same for sugar. There was no reduction as indicated by Fehling's test. B. The precipitate obtained was washed until no trace for sugar was found and until only a very slight trace of tannic acid remained. These tests were made by the usual Fehling and iron methods. The presence of diastase was always proved by the increase in the amount of sugar found by the Fehling or Bang determination, in the solution which was supposed to contain the diastase. The solutions were always incubated with an equal volume of a 1.% starch solution. Tests for sugar were made both before and after incubating. The increase in the amount of sugar found was the direct result of diastatic action upon the starch.

Processes D. and E.

After the processes A. B. and C. in the Acetone-Tannic acid method the products C from bacterial culture liquids, from the autolytic liver, and from the malted wheat extracts, were passed through the cellulose-aluminum phosphate prepared filter, the preparation of which will be given below. These were usually passed through twice to obtain complete adsorption if possible. This constituted the step D, in the process. The diastase may now be extracted with considerable difficulty from the cellulose-aluminum phosphate prepared filter, with either distilled water or a 50.% aqueous acetone solution, which is the step E, or the final treatment in the process. This

will be discussed more fully below.

4.1 Data from a Bacterial Culture Liquid.
Experiment Number 8-F-28.

20. Cc. of a bacterial culture liquid, prepared as explained previously, was treated with 5. cc. of albumin reagent, whose preparation was explained above, and 20. cc. of a 0.5% tannic acid reagent, prepared as also explained above. The solution was then centrifuged and the clear liquid decanted. A portion of the liquid remained which was separated from the precipitate by filtering it through a Schleicher and Schüll's number 595 filter paper. The entire amount of the precipitate thus obtained was treated with 20. cc. of a 50% aqueous acetone solution, and after digesting for a few minutes was tested for tannic acid by means of the iron test. Some tannic acid was found, so an additional 2 cc. of albumin reagent was added. The solution was now centrifuged and decanted and the liquid tested again for tannic acid by the iron test. Only a trace was found. The entire amount of the liquid was now evaporated to 10. cc. at room temperature, by means of an air current, and incubated at 50° C. for 17 hours. A Fehling's determination for sugar was then made which required 8.6 cc. of thiosulphate. The blank test at this time required 10.7 cc. of thiosulphate solution. According to Fehling's test for sugar, none was present when the above solution was placed in the incubator. From this experiment we find that diastase may be extracted from a bacterial culture liquid by the Acetone-Tannic Acid method.

4.2 Data from Autolytic Pig Liver.
Experiment Number 8-f-13.

This experiment was performed simply to test the use of the Acetone-Tannic Acid method for extracting diastase from autolytic liver.

40 C c. of the autolytic pig liver liquid, prepared as explained above, was treated with 20. c c. of a 0.5% solution of tannic acid, which also contained about 0.1% of acetic acid, and let stand at room temperature for a few minutes to precipitate the dissolved proteins. After the precipitate had settled the liquid was decanted, and the remaining portion containing the precipitate was filtered through a Schleicher and Schüll's number 595 filter paper, by means of slight pressure from the filter-pump. The filtrate which contained numerous non-colloidal decomposition products was rejected. The filter paper and the precipitate was placed in a flask with 40. c c. of a 50.% aqueous acetone solution, and shaken until the filter paper was broken into small bits, and the precipitate was thoroughly dissolved. The solution is now filtered as above described and the filtrate evaporated at 40°C. to 50°C. to remove the excess of acetone. As this is done a white possibly alcohol soluble protein precipitate appears, which is obtained by filtering as described above. The precipitate is made up to 10. c c. with distilled water and incubated at 37°C. with an equal volume of a 1.% starch solution. According to Fehling's test there was no sugar in the solution when placed in the incubator. After incubating a few hours there was an abundant reduction for sugar by Fehling's test. This experiment shows that diastase may be extracted from autolytic liver by the Acetone-Tannic Acid method.

Data from Malted Wheat Extract.
Experiment Number 8-1-20.

This experiment was performed to test the use of the Acetone-Tannic Acid method for extracting diastase from malted wheat.

10. Grams of malted wheat prepared as explained above, were treated with 50. c.c. of distilled water, and let digest for about one hour. 20. C.c. of this malted wheat extract was treated with an equal volume of the previously described tannic acid reagent. The solution was let stand for a short time and then filtered as previously described. The precipitate obtained was digested with 40. c.c. of a 50.% aqueous acetone solution and again filtered as above. The precipitate thus obtained was treated with 40.c.c. of a 0.5% starch solution and incubated at 37°C. for 18 hours, and a Fehling's test for sugar made which required 9.8 c.c. of thiosulphate solution. The filtrate obtained above was incubated with an equal volume of a 1.% starch solution under the same conditions and for the same length of time as the precipitate, and a Fehling's test for sugar was made which required 9.3 c.c. of thiosulphate. Fehling's test for sugar was made upon both of these solutions previous to incubation, with a negative result. The blank test upon Fehling's method required 10.5 c.c. of thiosulphate at the time the above tests were made.

In the above experiment the diastase was only partially extracted by the 50.% aqueous acetone solution. This is the final step in the Acetone-Tannic Acid method. The small result due to diastatic action in the above experiment is in a large part due to the great dilution of the original liquid.

5. Separation of Diastase from Adventitious Substances by the Adsorption Method.

A number of compounds are here used in the attempt to find an adsorbent of diastase, and at the same time to free it from its advantitious substances.

5.1 Adsorption by $Al_2(PO_4)_2$.

51.1 Apparatus Used.

In the process of separating diastase by adsorption the following apparatus and materials were used; A filter pump; a Scheicher and Schüll's number 595 filter paper, and and aqueous suspension of aluminum phosphate, $Al_2(PO_4)_2$.

51.2 Preparation of $Al_2(PO_4)_2$ Suspension.

150. Cc. of an $AlCl_3$ solution is precipitated with $\frac{1}{4}$ the volume of a 0.2 N. solution of NH_4PO_4. After thoroughly mixing let stand for some time and then decant and wash repeatedly with distilled water which has been slightly acidified with concentrated acetic acid. After this thorough washing the precipitate $Al_2(PO_4)_2$ is suspended in 500. cc. of distilled water. The amount of this suspension used in the preparatinn of the cellulose aluminum phosphate prepared filter will be given subsequently.

51.3 Preparation of the Cellulose-Aluminum Phosphate Filter.

The filter paper was 50.5 m. m. in diameter, placed in a porcelain Buchner's funnel, and thoroughly washed with water. Then the suspension of aluminum phosphate was poured on the filter paper and by means of the vacuum pump allowed to run through under slight pressure. As much aluminum phosphate suspension was used as was

necessary to form a coating on the filter paper of from one to two millimeters of $Al_2(PO_4)_2$.

The prepared filter should be dense enough to allow the filtrate to pass through not faster than about 30 drops per minute. This slow rate facilitates the process of adsorption.

Through the prepared filter were passed various kinds of solutions; a 50.% aqueous acetone extract of malted wheat; a 75.% aqueous acetone extract of malted wheat; a 50.% aqueous acetone extract of malted wheat diluted with distilled water; bacterial culture liquid which was previously treated with thymol water; and autolytic liver extract.

Data on most of this material will be given subsequently.

51.4 Data from Bacterial Culture Liquid.
Experiment Number 8-4-4-.

The direct adsorption of diastase by means of $Al_2(PO_4)_2$ was accomplished as follows: 10.Cc. of the original bacterial culture solution was treated with about 5. cc. of toluol-thymol solution and thoroughly shaken and allowed to stand for about 10 minutes. The bacteria must be dead in order that the diastase may be extracted, and this treatment is to kill them. The bacterial culture solution was now withdrawn from under the toluol-thymol solution by means of a pipette and twice passed through the prepared cellulose-aluminum phosphate filter. The filter was then washed with about 10. cc. of distilled water and all the adhering sugar removed, as indicated by the Fehling's test on the wash water. The prepared filter was now placed in a flask with 10. cc. of distilled water and shaken until the precipitate together with the filter paper was broken into small pieces. To this was added an equal volume of a 1.% starch solution and the whole was incubated at 50°C.

Before incubating, however, a test for sugar was made by Fehling's method and none was found. After incubating the above precipitate solution for two hours at 50°C., Fehling's test for sugar was again made and an abundant reduction was obtained. The filtrate from the above liquid was set with an equal volume of a 1.% starch solution and incubated for two hours at 50°C. and Fehling's test for sugar made with a negative result.

From the above experiment I conclude that diastase as found in bacterial liquid cultures may be extracted by means of the prepared cellulose aluminum phosphate filter.

Date on Malted Wheat.
Experiment Number C-3-27/1.

10. Cc. of the 50.% aqueous acetone extract of malted wheat, prepared as explained above, was diluted with 5. cc. of distilled water and passed through a cellulose-aluminum-phosphate filter. The prepared filter was now washed with distilled water until no adhering sugar remained, as determined by Fehling's test on the wash water. The prepared filter was now placed in a flask with 15.cc. of distilled water and shaken until it was broken into very fine particles. To this solution was added 15. cc. of a 1.% starch solution and incubated at 37°C. Before incubating a portion was tested for sugar by Fehling's method and none was found.

Table on Experiment C-3-27/1.

Cc. of $Na_2S_2O_3$ used in the Sugar Determination.		
Blank Reading	Reading after 2 hrs of 37°C. Incubation.	Difference due to Diastase
10.7 cc.	6.8 cc.	3.9 cc.

From the above table we see that the blank reading was

10.7 cc, and the determination upon the solution after 2½ hours of incubation was 6.3 cc. of sodium thiosulphate. The diastase action is equivalent to the amount of sugar produced during incubation and is represented by the difference between the initial and final readings or 3.9 cc. of sodium thiosulphate. The extract values can be found in Schoorl's published table, published Zeitschr. f. angew. Chem. Jahrg. 1899, pp. 677-685. There was no reduction for sugar by Fehling's test previous to incubation.

Experiment Number 8-3-27/2.

Another experiment was made which tested the cellulose-aluminum phosphate prepared filter upon a solution containing a larger percentage of acetone. To 10.0c. of a 70.% acetone extract of malted wheat, of the same material as that used in preceding experiment, was added 5. cc. of concentrated acetone. This solution was twice filtered through a cellulose-aluminum phosphate prepared filter, which was made by the method previously described. The adhering sugar was washed out with the same amount of distilled water as used in the preceding experiment. Tests for sugar were made on the wash water by Fehling's method. None was found. The prepared filter was now placed in a flask with 15. cc. of distilled water and shaken until the filter paper was broken into small particles. An equal volume of a 1.% starch solution was now added and before incubating a test for sugar was made by Fehling's method and there was none found. After the solution had incubated 2½ hours at 37°C. a second test for sugar was made by Fehling's method, which gave an abundant reduction. The amount of thiosulphate used was 6.4 cc. The blank test was 10.7 cc.

Table of Experiment Under
6-5-17/2.

Cc. of $Na_2S_2O_3$ used in the Sugar Determination.		
Blank Reading	Reading after 2½ hours of 37°C. Incubating.	Difference Due to Diastase.
10.7 cc	6.4 cc.	4.3 cc.

In the above table it will be noticed that the blank test required 10.7 cc. of $Na_2S_2O_3$ to equalize the copper. There was no reduction for sugar by Fehling's test previous to incubating but after 2½ hours incubating at 57°C. the above test for sugar required 6.4 cc. of $Na_2S_2O_3$. The difference 4.3 cc. being due to diastase action upon the starch. In comparing the above tables which were made from comparable experiments it appears that there is a difference of diastase action equivalent to only 0.4 cc. of $Na_2S_2O_3$ which is in favor of the solution having the higher percent of acetone.

51.7 Control Experiment.

A control on the proceding experiments was made as follows:- A cellulose-aluminum phosphate filter was prepared in the usual way, and washed with distilled water. It was then placed in a flask with distilled water and shaken to small particles and incubated with an equal volume of a 1.% starch solution. After incubating for 1 hour at 37.°C. a test for sugar was made by Fehling's method, and there was none found.

An experiment was also performed where a 50.% aqueous acetone solution was passed through a cellulose-aluminum phosphate prepared filter and after inculating as above gave no sugar reduction by Fehlings test.

5.2 Action of Zinc Compounds on Diastase and its Adsorption by Them.

52.1 Action of ZnO on Diastase. Experiment Number 8-4-28/1.

The mixture of zinc oxide used in this experiment was made by adding 10. grams of zinc oxide to 100. c.c. of distilled water. 5 C c. of 50.% acetone extract of wheat known to contain diastase and prepared as explained above, was diluted to 25. c.c. with distilled water. To this was added an equal volume of the above zinc mixture. These were thoroughly mixed and allowed to stand at room temperature for about 10 minutes. The solution was then centrifuged and the precipitate washed free from adhering sugar, as proved by testing the wash water for sugar by Fehling's method. 10. c.c. of the clear centrifugate was incubated with an equal volume of a 1.% starch solution, for one hour at 50°C. A Fehling's test for sugar was made before incubating, which required 3.0 c.c. of the thiosulphate solution to equalize the copper. After incubating, a Fehling's test was again made for sugar which required 2.9 c.c. of thiosulphate solution to equalize the remaining copper. A test was also made upon the precipitate. 1/5 of the precipitate obtained by centrifuging was made up to 10. c.c. with distilled water and incubated with an equal volume of a 1.% starch solution. According to Fehling's test there was no sugar present when placed in incubator. After 1 hour Fehling's test was again made with a negative result. From the above experiment I conclude that zinc oxide as used inhibits diastatic action.

52.2 Action of $ZnSO_4$ on Diastase Adsorbed by a Cellulose-Aluminum Phosphate Filter
Experiment Number 8-4-23/1.

This experiment was performed in order to learn the affect of a zinc-sulphate solution upon the diastase of a prepared cellulose-aluminum phosphate filter. 250 c. c. of a 50.% acetone extract of wheat was passed through a cellulose-aluminum phosphate filter, as described above. The adhering sugar was washed from the prepared filter with distilled water, as proved by testing the wash water for sugar by Fehling's method. The prepared filter supposed to contain the diastase was divided equally into two parts. One part was treated in a flask with 5.c c. of an 11.% zinc sulphate solution for 48 hours. At the end of this time 5.c c. of distilled water was added, and also 5.c c. of a 1.% starch solution, and the entire amount incubated for 5 hours at 50°C. There was no sugar according to Fehling's test when the solution was placed in the incubator. After incubation, Bang's test for sugar, in the customary proportions, required 3.2 c c. of hydroxylamine solution to equalize the copper present. A blank test was also made on Bang's determination, which required 3.5 c c. of hydroxylamine solution to equalize the copper.

The other half of the prepared filter was placed in a flask containing 5.c c. of distilled water. After standing at room temperature for 48 hours, 5.c c. of distilled water and 5.c c. of a 1.% starch solution were added. The solutions were then thoroughly mixed and a test for sugar by Fehling's method made, with a negative result. The solution was then incubated for 5 hours at 50°C. and then a Bang's sugar test with the customary proportions

was made which required 2.6 c c. of hydroxylamine to equalize the remaining copper. A blank test was again made on Bang's determination, which required 3.5 c c. of hydroxylamine solution to equalize the copper.

The above experiment shows that diastase, as adsorbed by the prepared filter, is denatured when treated with an 11.% zinc sulphate solution for any length of time.

522.1 Concentration of $ZnSO_4$ Which Inhibits-Diastatic Action.
Experiment Number 8-5-1.

In the preceding experiment, 8-4-28/1, it was found that an 11.% zinc sulphate solution would inhibit diastatic action. The present experiment is to test the effect of very weak solutions of zinc sulphate on diastase. It was found by experiment, which will be explained below, that a suspension of lead phosphate is an excellent adsorbent of diastase. This fact was taken advantage of in this experiment.

To 10. c c. of a 50.% acetone extract of wheat was added 20. c c. $Pb_3(PO_4)_2$ suspension. These were thoroughly mixed and allowed to settle three or four times. The liquid was then centrifuged and the precipitate divided into three equal parts. Each was tested as follows. 1/3 Of the precipitate was made up to 5. c c. with distilled water. To this was added 5. c c. of a 2.% zinc sulphate solution, and the entire amount incubated with 10. c c. of a 1.% starch solution. The initial test for starch, taken by Bang's method in the usual proportions, before incubating was 2.9 c c. of hydroxylamine solution. After incubating 18 hours at 50°C. the sugar determination by Bang's method as usually performed was again

taken, which required 2.7 c c. hydroxylamine solution to equalize the remaining copper. Another 1/3 of the lead phosphate containing the diastase was made up to 5.c c. with distilled water. To this was added 5. c c. of a 4.% zinc sulphate solution and the entire amount incubated with 10 c c. of a 1.% starch solution. The initial test for sugar taken according to Bang's method required 3.0 c c. of hydroxylamine solution. After incubating at 50°C. for 18 hours the customary Bang's determination for sugar was again made, which required 2.8 c c. of hydroxylamine solution.

The remaining 1/3 of the lead phosphate which contained the diastase was made up to 5. c c. with distilled water. To this was added 5. c c. of distilled water, and the entire amount incubated with 10. c c. of a 1.% starch solution. Before incubating, the usual Bang's determination for sugar was made, which required 2.9 c.c. hydroxylamine solution. After incubating for 18 hours at 50°C. the test for sugar was again made according to Bang's method. This time it required 2.3 c c. of hydroxylamine solution to equalize the remaining copper color.

From these experiments the conclusion is drawn that all zinc sulphate solutions inhibit diastatic action.

52.3 $Zn_3(PO_4)_2$ as Direct Adsorbent of Diastase.

Experiment Number 8-4-28/2.

The zinc phosphate used in this experiment was made by precipitation from zinc sulphate and sodium phosphate (Na_2HPO_4). The precipitate was washed with distilled water. 5. C c. of a 50.% acetone extract of malted wheat was diluted to 25. c c. with distilled water. To this was added an equal volume of the above zinc phosphate solution. This was thoroughly mixed and allowed to settle three or four times. The solution was then centrifuged, and to 10. c c. of the clear liquid was added 10. c c. of a 1.% starch solution. A test for sugar was made in the usual proportions by Bang's method, which required 2.5 c c. of hydroxylamine solution to discharge the copper color. The solution was then incubated at 50°C. for one hour, and Bang's sugar test was again made, which required 1.6 c c. of hydroxylamine. 1/5 Of the precipitate was made up to 10. c c. with distilled water and set with an equal volume of a 1.% starch solution. A Fehling's test for sugar was then made, with a negative result. After incubating at 50°C. for one hour, Fehling's test for sugar was again made, with a negative result.

Experiment Number 8-4-28/3.

In the experiment 8-4-28/2 we found that the zinc phosphate adsorbed no diastase as indicated by one hour's incubation. This experiment was performed on some more of the same 50.% aqueous acetone extract of malted wheat. Some of the same zinc phosphate was also used. The experiments were made as nearly alike as possible, the time of incubation alone being changed. 5. C c. of the

50.% acetone extract of malted wheat was made up to 25. c c. with distilled water. To this was added 25. c c. of the zinc phosphate solution and thoroughly mixed and allowed to settle three or four times. The solution was then centrifuged and 1/5 of the precipitate was made up to 10. c c. with distilled water and 10. c c. of a 1.% starch solution was added and after mixing, Bang's test for sugar was made. 3.4 c c. of hydroxylamine solution were necessary to ~~equaline~~ remove the copper color. The solution was set away over night at room temperature, and incubated the next morning at 50°C. for three hours. Bang's sugar test was then made and 2.6 c c. of hydroxylamine solution were required. 10. c c. of the centrifugate was set with 10. c c. of a 1.% starch solution and Bang's sugar test was made which in the usual proportions required 2.1 c c. of hydroxylamine. After setting over night at room temperature the solution was incubated at 50°C. for three hours and then Bang's sugar test was again made. This time it required 1.8 c c. of hydroxylamine.

A control test was made as follows;- Equal volumes of the zinc phosphate and starch solutions as used in previous experiments were incubated together. Bang's sugar test before incubating required, 3.6 c c. of hydroxylamine and after standing over night at room temperature and incubating three hours the next morning at 50°C., the same as preceding experiments, Bang's sugar test, again made, required 3.6 c c. of hydroxylamine.

From the above experiments we find that the change of the amount of sugar during incubation, due to diastatic action in the precipitate, is equal to 0.8 c c. of hydroxylamine solution, while the change in the amount of sugar in the centrifugate, during the same incubation, is represented by only 0.3 c c. of hydroxylamine

solution. The same amounts of the original diastatic solution are represented in each of the above incubations. Hence we find that 8/11 of the diastase in the original solution was adsorbed by the zinc phosphate used.

5.3 Adsorption and Acceleration of Diastase by $Pb_3(PO_4)_2$.

53.1 Direct Adsorption by $Pb_3(PO_4)_2$. Experiment Number 8-5-1/N.

This experiment was performed upon an extract of malted wheat to test the adsorbing power of a $Pb_3(PO_4)_2$ suspension, when in direct contact with the diastatic liquid. The $Pb_3(PO_4)_2$ suspension used was prepared as follows;- 100. C c. each of a 0.5 m. solution of $Na_2H(PO_4)$; and 0.3 m. solution of $Pb_n(C_2H_3O_2)_m$ were thoroughly mixed and washed repeatedly with distilled water by decantation and finally the suspended $Pb_3(PO_4)_2$ was made to a volume of 500. c c. with distilled water. 75. Grams of malted wheat were ground and treated with 75. c c. of a 50. % aqueous acetone solution. Two days after, 10. c c. of the clear supernatant liquid was withdrawn by means of a pipette and mixed with 10. c c. of the above lead phosphate suspension. Upon settling and agitating several times, the mixture was centrifuged and the liquid poured off. The residue was suspended in a volume of 10. c c. of distilled water and 10. c c. of a 1.% solution of Kahlbaum's soluble starch added. The entire amount was mixed and a few cubic centimeters was filtered through a paper filter. 1. C c. of the filtrate and 5. c c. of Bang's $CuSO_4$ solution was boiled three minutes, rapidly cooled and then titrated with Bang's hydroxylamine. 2.7 C c. were necessary

to discharge the color of the copper solution. The remaining unfiltered portion of the liquid was incubated at 50°C. After 40 minutes another 1. c c. was taken out as above described and likewise titrated. There was required 2.4 c c. of hydroxylamine to discharge the copper color in the solution. Hence diastase produced sugar in 40 minutes equivalent to the copper represented by the 0.3 c c. of the hydroxylamine solution. The fermentation tests were conducted upon solutions of twice the volume of the original raw material. A blank experiment was made as follows;- 5. c c. of Bang's $CuSO_4$ solution was titrated with Bang's hydroxylamine solution and 3. c c. was required to neutralize the copper color, boiling being omitted.

53.8 Accelerating Effect of $Pb_3(PO_4)_2$.
Experiment Number 8-5-2/1.

This experiment was performed to determine the effect of lead phosphate upon diastase.

To 4. c c. of a 50.% aqueous acetone extract of malted wheat was added 6. c c. of lead phosphate suspension, prepared as previously described, and after thoroughly mixing and allowing to settle for about 15 minutes was incubated with 10. c c. of a 1.% starch solution. Before incubating however a Bang's test for sugar was made, which required 2. 0 c c. of hydroxylamine to remove the copper color. After incubating for about 48 hours at 50°C. Bang's test for sugar was again made. It now required 0.4 c c. of the hydroxylamine solution to equalize the copper. In connection with this experiment a control was also made as follows;- 4. 0 c. of the same 50.% aqueous acetone extract of malted wheat as used in experiment 8-5-2/1, was made up to 10. c c. with distilled water.

This entire amount was incubated with 10. c c. of 1.% starch solution. Before incubating a test for sugar was made by Bang's method which required 1. 8 c c. of hydroxylamine solution to decolorize the remaining copper. After incubating 48 hours at 50°C. Bang's sugar test was again made which now required 0.9 c c. of hydroxylamine to decolorize the remaining copper.

Table on Experiment Number 8-5-8/1.

Composition	Sugar determination in c c.of hydroxylamine				
	Before Incubating	48 hours 50°C.incubating	Increase	due to diastase	Acceleration
4.c c. Extract of wheat 10.c c. of a 1.% starch sol. 6.c c. $Pb_3(PO_4)_2$	2.0 c c.	0.4 c c.	1.6 c c.	0.9 c c.	0.7 c c.
4.c c. Extract of wheat 10.c c.of a 1.% starch sol. 6.c c. Dist H_2O.	1.8 c c.	0.9 c c.	0.9 c c.	0.9 c c.	

In the third column of the above table we find that where lead phosphate was present we had an increase of sugar during 48 hours of incubation at 50.°C.;equal to 1.6 c.c. of hydroxylamine, while under the same conditions where no lead phosphate was present we have a change represented by only 0.9 c c. of hydroxylamine solution. Hence I conclude that the lead phosphate has accelerated diastatic action during the 48 hours of incubation at 50°C., equal to the amount which is represented by 0.7 c c. of hydroxylamine as indicated in the last column of the above table.

5.4 Comparison of Adsorption by $Zn_3(PO_4)_2$ and $Pb_3(PO_4)_2$.

In the preceding experiments it has been shown that a zinc sulphate solution, even as weak as 0.5% inhibits diastatic action to a marked degree; also that lead phosphate as an adsorbent does not inhibit, but rather accelerates, diastatic action. The following experiments are for the purpose of comparing the effect of zinc phosphate and lead phosphate upon a common diastatic liquid.

Data on Experiment
Number 8-4-30/1.

5.cc. of 50% aqueous acetone extract of malted wheat.

20,cc. of----------distilled water.

25.cc. of----------zinc phosphate.

Experiment	1.% starch Solution	Amount of Hydroxylamine		
		Initial Reading	Final Reading	Difference
1/5 of centrifugate or 10. cc.	10.cc.	1.2 cc.	0.7 cc.	0.5 cc.
1/5 of Precipitate and H_2O 10.cc.	10.cc.	3.0 cc.	2.7cc.	0.3 cc.

Experiment Number 8-4-30/1.

To 5 cc. of a 50.% aqueous acetone extract of malted wheat was added 20. cc. of distilled water and to this was added 25. cc. of a zinc phosphate suspension, prepared as previously explained. This mixture was allowed to digest for a few minutes, and then it was centrifuged. 10. Cc. of the centrifugate was made up with an equal volume of a 1.% starch solution, and filtering a portion an estimation of sugar was made by Pang's method which required

0.7 cc. of hydroxylamine solution. 1/5 of the residue obtained when centrifuged was made up to 10. cc. with distilled water, and incubated with an equal volume of a 1.% starch solution. Before incubating a small portion was filtered and a sugar determination by Bang's method made, which required 3.0 cc. of hydroxylamine solution. After incubating the unfiltered portion of the solution for 14 hours at 50°C. Bang's sugar estimation was again made. This time it required 2.7 cc. of hydroxylamine to remove the copper color. In this experiment we had diastase adsorbed by 1/5 of the zinc phosphate which acted upon the starch and converted it into sugar which is represented by the difference in the amounts of hydroxylamine used in titrating or 0.3 cc. In the centrifugate we had diastatic action equal to the increase in amount of sugar during incubation and is represented by the difference in the amounts of hydroxylamine used, or 0.5 cc. Thus we see that the zinc phosphate failed to adsorb half of the diastase in the original liquid.

Data on Experiment
Number 8-4-29/2.

5. cc. 50.% aqueous acetone extract of Malted Wheat.
20. cc. of----------distilled water.
25. cc. of----------lead phosphate.

Experiment	1.% starch solution	Amount of Hydroxylamine		
		Initial Reading	Final Reading	Difference
1/5 of Centrifugate or 10 cc.	10. cc.	1.7 cc.	0.7 cc.	1.0 cc.
1/5 of Precipitate and H_2O 10. cc.	10. cc.	2.8 cc.	1.4 cc.	1.4 cc.

Experiment Number 8-4-29/2.

To 5. cc. of a 50.% aqueous acetone extract of malted wheat, some of the same as used in the experiment 8-4-30/1, was added 20. cc. of distilled water, and to this was added 25. cc. of a lead phosphate suspension, made as explained above. After agitating and letting stand for a few minutes the solution was centrifuged. 10. Cc. of the centrifugate was made up with an equal volume of a 1.% starch solution, same as that used in the experiment 8-4-30/1, and, after taking a sugar determination by Bang's method, which required 1.7 cc. of hydroxylamine, was incubated at 50°C. for 14 hours. A Bang's sugar estimation was then again made, which required 0.7 cc. of hydroxylamine solution to equalize the remaining copper color. One fifth of the residue obtained by centrifuging was made up to 10. cc. with distilled water and after mixing with an equal volume of a 1.% starch solution, same as used above, was tested for sugar by Bang's method which required 2.8 cc. of hydroxylamine solution. After incubating 14 hours at 50°C. Bang's sugar determination was again made upon a portion of the filtered liquid of the above solution. It now required 1.4 cc. of the hydroxylamine solution to remove the copper color.

In this experiment we also find that only a small portion of the diastase was adsorbed from the original extract.

In the preceding experiments the adsorbents acted upon the diastatic material practically the same length of time, which was about 20 minutes.

In the experiment where zinc phosphate was used we had diastase adsorbed by 1/5 of the precipitate which produced sugar to the amount indicated by 0.3 cc. of hydroxylamine, while in the

experiment where lead phosphate was used as an adsorbant we had diastase adsorbed which produced sugar equivalent to 1.4 cc. of hydroxylamine solution.

The conclusion from the results is that lead phosphate is a much better adsorbent of diastase than zinc phosphate.

54.1 Factors in the Selection of the Best Method.

In the selection of the best method of adsorption, the most important consideration is that of its efficiency in yielding the largest amount of diastase in its purest form. Furthermore only such adsorption substances can be used as do n t inhibit diastase action. The adsorbing material should also be easily removable from the mixture by means of the centrifuge or possibly filtration. If a compound like, e.g., lead phosphate even accelerates diastase, this property may be considered a distinct advantage. Some further examples will be presented which deal with various aspects, especially that of efficiency, which would be determining factors in the selection of a method.

5.5 Efficiency of Lead Phosphate as Adsorbent.
Experiment Number 8-5-5/1.

10 Cc. of a 5% acetone extract of malted wheat and 10. cc. of a lead phosphate suspension, prepared as previously explained, were thoroughly mixed and allowed to settle two or three times. The solution was now centrifuged and the precipitate obtained was made up to 10 cc. with distilled water and incubated at 50°C. Before incubation a test for sugar was made by Bang's method, which required 3.0 cc. of hydroxylamine solution to be equivalent to the copper remaining. This was designated as precipitate number I. The centrifugate obtained above was treated with the solid material obtained from 10 cc. of the lead phosphate suspension, and the whole made up to 20 cc. with distilled water. This solution was thoroughly mixed and allowed to settle two or three times, and then centrifuged. The precipitate thus obtained was made up to 10 cc. with distilled water and incubated at 50°C. with 10 cc. of a 1% starch solution. Before incubating a test for sugar was made by Bang's method, which required 3.5 cc. of hydroxylamine solution. This was designated as precipitate number II.

The centrifugate obtained above was set with an equal volume of a 1% starch solution, which was 15 cc. After thoroughly mixing, a test for sugar by Bang's method was made which required 2.7 cc. of hydroxylamine solution. The centrifugate containing the starch solution was now placed in the 50°C. incubator. This solution was designated as centrifugate or filtrate number II.

After 18 hours incubation tests for sugar were made by Fang's method upon each of the three solutions and the results were as given in the following table.

Table showing the efficiency of $Pb_3(PO_4)_2$ as an adsorbent of diastase.

	Amount of Hydroxylamine			
	Initial Reading	After 18 hours of incubation	After 84 hours of incubation	Difference after incubating
Precipitate Number I	3.0 cc.	2.0 cc.	2.0 cc.	1.0 cc.
Precipitate number II	3.2 cc.	3.0 cc.	3.0 cc.	0.2 cc.
Filtrate number II	2.7 cc.	2.7 cc.	2.7 cc.	

In the preceding table we find that in the first treatment the diastase was adsorbed sufficiently to produce the change in the amount of sugar represented by 1.0 cc. of hydroxylamine. The diastatic action took place during the first 18 hours of incubation. No further change in the amount of sugar was produced after incubating 42 hours.

In the second treatment diastase was adsorbed sufficient to produce the change in the amount of sugar represented by 0.2 cc. of hydroxylamine. Here again all the diastatic action took place upon the first 18 hours of incubation.

In the tests upon the filtrate no diastatic action was obtained. According to the above experiments 5/6 of the total amount of diastase was adsorbed during the first treatment.

55.1 Adsorption of Dilute Diastase by $Pb_3(PO_4)_2$.
Experiment Number 8-5-13/2.

This experiment was performed for the purpose of testing lead phosphate as an adsorbent upon a very dilute diastatic solution.

One cubic centimeter of a 50.% aqueous acetone extract of malted wheat was diluted to 100. cc. with distilled water. To this was immediately added 10. cc. of the above described, lead phosphate suspension. This liquid was thoroughly mixed and allowed to settle three or four different times. The liquid was now decanted and the precipitate was made up to 10. cc. with distilled water. To this was added 10. cc. of a 1.% starch solution and a Bang's sugar determination immediately taken.

Bang's Determination for Sugar in

cc. of Hydroxylamine.

Initial Reading	Reading After Incubating at 50.°C.		
	3 hours	5 hours 30 minutes,	23 hours
3.5 cc.	3.7 cc.	3.6 cc.	3.6 cc.

The initial determination for sugar was 3.5 cc. of hydroxylamine as indicated in the first column of the above table. After incubating at 50°C. for 3 hours the second sugar determination was made as above with a result of 3.7 cc. of hydroxylamine being required to equalize the copper. After 5 hours and 30 minutes incubation, another determination was made, as above which required 3.6 cc. of hydroxylamine. At the end of 23 hours incubation at 50°C. a sugar determination was again made by Bang's method which required 3.6 cc. of hydroxylamine. During the first three hours of incubation there was a diastatic action equal to 0.6 cc. of hydroxylamine. During the first 5½ hours the diastatic action was equal to 0.9 cc. of hydroxylamine, which also represents the entire diastatic action.

The conclusion is drawn from this experiment that lead phosphate will adsorb diastase from a very dilute diastatic solution,

and that the most of the diastatic action takes place during the first few hours of incubation.

55.2 Adsorption of Diastase from Bacterial Liquid Culture by $Pb_3(PO_4)_2$. Experiment Number 8-5-13/3.

This experiment was performed for the purpose of testing lead phosphate as an adsorbent of diastase from bacterial liquid cultures. A portion of a bacterial liquid culture which was three days old was treated and thoroughly mixed with a toluol-thymol solution for about 10 minutes. 10 Cc. of the bacterial liquid was withdrawn from underneath the toluol-thymol by means of a pipette and treated with 10.cc. of lead phosphate suspension, prepared as described previously. This liquid was mixed thoroughly and allowed to settle three or four times. The clear solution was now decanted and the precipitate made up to 10. cc. with distilled water, and set with 10. cc. of a 1.% starch solution. Pang's sugar determination was now made which required 3.5 cc. of hydroxylamine. After incubating at 50.°C. for about 20 hours the sugar determination was again made, as above, which required 3.2 cc. of hydroxylamine. From the above experiment I conclude that diastase as found in a bacterial liquid culture can be extracted by means of lead phosphate.

5.6 Extraction from $Pb_3(PO_4)_2$ Adsorbent of Diastase.

Diastase which has been adsorbed by lead phosphate may be extracted in a number of different ways. The following experiments present the usual means which were employed, and is confined to the

A. D. E. Process.

56.1 Extraction with H_2O.
Experiment Number 8-4-24/2.

A. A 50.% aqueous acetone extract of malted wheat was prepared as previously explained, and 5. cc. of this extract was diluted to 25. cc. with distilled water. D. The above liquid was treated with an equal volume of lead phosphate suspension, prepared as previously described, and was thoroughly mixed and allowed to settle three or four times. This required about fifteen to twenty minutes time. The clear liquid was then siphoned off and the residue washed with about 20. cc. of distilled water three different times, to remove the adhering sugar. E. The lead phosphate was now made up to 20. cc. with distilled water and set away at room temperature to extract for 14 hours. The clear liquid from the original solution obtained by siphoning, as mentioned above, was set with equal volumes of a 1.% starch solution and incubated at 50.° C. for one hour. A Fehling's test for sugar was then made, with a negative result.

A portion of the lead phosphate after standing with distilled water, as mentioned above, was filtered and the filtrate set with an equal volume of a 1.% starch solution. A Fehling's test for sugar was immediately made with a negative result. After standing at room temperature only about 10 minutes Fehling's test for sugar was again made, and an abundant reduction was obtained.

The conclusion is drawn that water will extract diastase almost completely in 14 hours, when adsorbed by lead phosphate.

56.2 Extraction with Phosphoric Acid.
Experiment Number 8-4-25/1.

A. 10. cc. of a 50.% aqueous acetone extract of malted wheat, prepared as explained above, was diluted with an equal volume of distilled water, D. To this solution was added 50. cc. of a lead phosphate suspension, made as previously described, and thoroughly mixed and allowed to settle three or four times. The clear liquid was then siphoned off and the residue washed three or four times with about 20. cc. of distilled water. Finally the lead phosphate was made up to 50. cc. with distilled water, and 20. cc. of a 0.002% phosphoric acid was added and the entire amount set away at room temperature for two days. 10. Cc. of the clear liquid were then taken and incubated with an equal volume of a 1.% starch solution at 50°C. for three hours. A Pang's sugar test was then made which gave a negative result.

The lead phosphate precipitate was made up with distilled water and also incubated with an equal volume of a 1.% starch solution. Before incubating, a Fehling's test for sugar was made which gave a negative result. After incubating for one hour at 50°C., Fehling's test was again made upon the above solution, and an abundant reduction was obtained.

The conclusion is drawn from this experiment that a 0.001% phosphoric acid solution fails to extract diastase when it is adsorbed by lead phosphate.

56.3 Extraction with a 50.% Acetone Solution.
Experiment Number 8-5-18/1.

In connection with this experiment a control with water as an extractant was also conducted, and the data obtained from it will also be given in order that a direct comparison may be made.

A. A 50.% aqueous acetone extract of malted wheat was prepared as above described and when five days old 30. cc. of the liquid was removed. D. This liquid was treated with 30. cc. of the lead phosphate suspension, already described, and thoroughly mixed and let settle three or four different times. Then the liquid was siphoned off and the residue washed with distilled water until all the adhering sugar was removed, as indicated by Fehling's test upon the wash water. The residue was then made up to 30. cc. with distilled water and after thoroughly mixing was immediately divided into two equal parts, which were distinguished as number I and II. E. The mixture number I was treated with an equal volume 15. cc. of a concentrated acetone solution, and was thoroughly mixed and, after digesting for two hours at room temperature, was again thoroughly mixed and 10. cc. filtered through a Scheicher and Schüll's number 595 filter paper. The clear filtrate was set with an equal volume of a 1.% starch solution, prepared as previously described, and, after thoroughly mixing, a test for sugar by Pang's method was made, which required 3.2 cc. of hydroxylamine solution. The solution was now incubated at 50°C. for 18 hours and a test for sugar again made by Pang's method which required 2. 9 cc. of hydroxylamine solution. The solution just tested was designated as Ia.

The other half of the lead phosphate suspension designated as number II was treated with an equal volume of distilled water and thoroughly mixed and, after digesting for two hours, was again thoroughly mixed and 10. cc. removed and filtered, as above described, and the filtrate set with 10. cc. of a 1.% starch solution, and then tested for sugar by Pang's method. 3.2 Cc. of hydroxylamine

solution were required. This solution was designated as solution number IIa, and after incubating at 50°C. for 12 hours a test for sugar was again made by Pang's method which required 2.2 cc. of hydroxylamine solution.

After the 50% acetone had acted upon the lead phosphate precipitate for 14 hours, 10. cc. was set with an equal volume of a 1% starch solution, and after thoroughly mixing a portion was filtered and the filtrate tested for sugar by Pang's method. 2.2 Cc. of hydroxylamine was necessary to reduce the remaining copper. The solution which was set with starch was designated as number Ib, and was then incubated at 50°C. for 40 hours. A test for sugar was then made by Pang's method, which required 2.7 cc. of hydroxylamine solution. After the distilled water had acted upon the lead phosphate precipitate which is distinguished as number II, for 10 hours, the liquid was thoroughly mixed and 10.cc. removed. This was set with an equal volume of a 1% starch solution, and after a portion was filtered, the filtrate was tested for sugar by Pang's method. 3.0 cc. of hydroxylamine were required for this purpose. The solution which was set with the 1% starch was designated as number IIb, and incubated at 50°C. for 40 hours and again tested for sugar by Pang's method, as previously described. 2.3 Cc. of hydroxylamine were required to remove the copper color.

From the above experiments we see that a 50% acetone solution will extract diastase when it is adsorbed by lead phosphate. In the control experiment, which was performed under the same conditions, we find that distilled water extracts diastase even faster than the 50% acetone. This fact is clearly shown in both tests upon the extracting solutions.

I therefore conclude that distilled water is a better extractant than a 50% acetone solution, when the diastase is adsorbed by lead phosphate.

5.7 Summary of Adsorption Processes.

In the preceding experiments a number of examples have been given where zinc and lead compounds have been used as adsorbents of diastase. The aluminum phosphate was used more as a preparation for perfecting the filter than as a direct adsorbent, even though the latter quality no doubt added much to the efficiency of the prepared filter. The method of direct adsorption without filtration is much easier and more convenient than either the filtration or the acetone tannic acid method.

In the case of the cellulose-aluminum phosphate prepared filter, the precipitate was difficult to centrifuge. The insoluble zinc compounds show the property of adsorbing diastase, but those which are soluble were found to inhibit, and in some cases practically stop, diastatic action;

In the experiment under the head of comparison and adsorption by $Zn_3(PO_4)_2$ and $Pb_3(PO_4)_2$ it was found that the lead phosphate was the better adsorbent of diastase. It has also been shown that lead phosphate accelerates diastase action, which is also an advantageous property. Lead phosphate was also found to be very efficient as an adsorbent when submitted to various kinds of diastatic solutions.

Therefore the process of adsorption with $Pb_3(PO_4)_2$ from the aqueous or acetone solution is recommended for extracting and concentrating diastase.

C. The Properties Of The Diastatic Product E, Prepared By Processes A. D. E.

C.1 Continued Preservation Of Diastase. Experiment Number 8-4-6/2.

In this experiment the diastatic liquid E, with water as an extracting agent was prepared from 50.% aqueous acetone extract of malted wheat, an previously explained by the A.D.E. steps using the A.D.E. Acetone- Tannic acid combined with adsorption by lead phosphate method. This solution was set away at room temperature and after seven days a portion was incubated with equal volumes of a 1.% starch solution at 50.°C. for 2½ hours and an abundant reduction was obtained by Fehling's test for sugar. No sugar was present according to Fehling's test when the solution was placed in the incubator.

A prepared filter was incubated alone with a 0.5% starch solution at 50.°C. and there was no reduction of Fehling's solution when tested for sugar.

C.2 Residue Upon Evaporation. Experiment Number 8-4-24/1.

An E liquid was prepared with water as an extracting agent by the A. D. E. steps as previously explained using $Pb_3(PO_4)_2$, from a 50.% aqueous acetone extract of malted wheat. A portion of the clear E liquid was found to contain diastase by the usual incubation process, preceded and followed by Fehling's sugar tests. 10.Cc. of some of the same diastase liquid, as used in the above test, was evaporated to dryness at 40°C. to 50°C. by means of an air current, and a very small amount of a white residue remained. A control was also made by evaporating 10. cc. of a water extract from a prepared filter, which was prepared the same as the one used in

the above experiment, and a small residue also remained. A portion was also incubated for diastase and tested in the usual way, and no sugar was found in either test by Fehling's method.

This experiment shows that the small amount of residue obtained was not the diastase but rather came from the particles of the prepared filter which was used.

6.3 Protein Reaction Of The Prepared Diastase.
Experiment Number 6-4-15/1.

10. Cc. of an aqueous acetone extract of malted wheat, prepared as previously explained, was passed twice through a cellulose-aluminum phosphate prepared filter, also previously described, and the precipitate washed with distilled water while on the filter paper until no test for sugar was obtained in the wash water by Fehling's method. The first 10. cc. of wash water was incubated for 1 hour at 50°C. with a 1.% starch solution and then a test for sugar was made by Fehling's test with a negative result. A portion of the filter paper was also tested for diastase by thoroughly shaking and breaking it up with 10. cc. of distilled water and incubating with an equal volume of a 1.% starch solution at 50°C. for 1 hour and a good reduction was then obtained by Fehling's test. There was no reduction by Fehling's test just before the solution was placed in the incubator. Some of this solution known to be diastically active was tested for proteins by the Biuret test. A small amount was placed in a test tube with strong NaOH and then treated with a dilute solution of $CuSO_4$. There was no violet color produced, which proves the absence of any of the more complex proteins. From this test I conclude that diastase is not an ordinary protein.

6.4 . Carbohydrate Reaction Of Prepared Diastase.

Some of the liquid T was prepared with water as an extracting medium, as previously explained. A portion of the clear liquid, known to contain diastase, was boiled with dilute Fehling's solution and there was no reduction.

From this I conclude that diastase is not, or does not contain, a reducible carbohydrate.

7.1 The Action Of Asparagin On Diastase.
Experiment Number 7-11-15.

This experiment was performed for the purpose of determining the affect of asparagin on diastase. There were three different parts to this experiment, test, first control, and second control. The test part of the experiment was made up as follows:- 30. cc. of a 1.% asparagin solution; 50. cc. of a bacterial culture liquid, prepared as explained previously; 120. cc. of a 1.% starch solution. The first control consisted of 30. cc. of distilled water; 50. cc. of some of the same bacterial culture liquid as used in the test part of this experiment; 120. cc. of a 1.% starch solution. The second control consisted of; 30. cc. of distilled water; 50. cc. of some of the same as the above bacterial culture liquid; 120. cc. of a 1.% starch solution. Each of the parts to this experiment was made up with about 5. cc. of a solution of toluol and chloroform as antiseptic. Two different kinds of sugar determinations were made upon these solutions. First will be given the polarimetric determinations and then the Fehling's.

Polarimetric Determinations.
Data on Experiment 7-11-15.

Results in Degrees.			
Date	Test	First Control	Second Control
7-11-15	181.37°	181.35°	181.31°
7-11-16	181.07°	181.11°	181.76°
7-11-18	180.93°	181.08°	181.07°
7-11-19	180.77°	181.90°	181.05°
Diff. first interval	-.50°	-.14°	+.05°
Diff. second interval	-.14°	-.09°	-.34°
Diff. third interval	-.16°	-.06°	+.08°
Total Difference	-.80°	-.29°	

The day the above solutions were set and before incubation, the polarimetric-determination for sugar was made upon each. About 15. cc. of each of the above solutions was filtered through an asbestos filter, and about 15. cc. of the clear filtrate placed in the 2. decimeter tube of the polarimeter and about 10 readings for each solution taken and the average determined. The results for the first day, 7-11-15, were as indicated by the above table 181.37°, 181.35° and 181.31° for the test, first control and the second control respectively. The original prepared solutions were incubated at about 37° and polarimetric determinations for sugar made nearly every day as indicated by the above results.

It should be observed that in the second column of the above table, where the asparagin was present the total difference due to diastase action stands -.80°, to -.71° where no asparagin was present.

In the last column of the above table where the solution tested contained the bacteria which had been boiled, we find comparatively small changes, which are first positive and then negative. These changes are not altogether understood, but it is known by this and other experiments that boiling kills diastase. The main point of the above experiment is that the asparagin accelerates diastatic action.

Fehling's Determination for Sugar.

Date	Test	1st, Cont.	2nd. Cont.
7-11-16	1.70	1.84	No test made
7-11-18	1.78	2.65	" " "
7-11-19	2.99	2.07	" " "
Total Differences	1.29	1.13	" " "

A portion of the same filtrate upon which the above polarimetric determinations were made was afterwards tested for sugar by Fehling's method with the above results. Again in the first column we see the greater diastatic action. This solution contains the asparagin. So by this method of determinations also asparagin accelerates diastatic action.

The Action Of Tannic Acid On Diastase
Experiment Number 8-4-14/2.

15. Cc. of a 50% aqueous acetone extract of malted wheat was passed twice through a prepared cellulose-aluminum phosphate filter. The prepared filter was now divided into two equal parts.

Table On Experiment Number 8-4-14/2.

Digested 18 hours	Amount H_2O added	Added 0.3% Tan. Acid.	Added 1% Starch Solution.	Initial Sugar test.	Reduction of Diffeh after incubating at 50°C. for 4 hrs. 30 min.
½ of filter + 5 cc. Dist. H_2O.	15 cc.	0	10 cc.	None	large
½ of filter + 5 cc. of 0.3% Tan. Acid.	10 cc.	5 cc.	10 cc.	None	slight.

In the above table it should be observed that one half of the filter paper was treated with distilled water while the other half was treated with 0.3% tannic acid for 18 hours, and afterwards made up to the same volume with water and tannic acid as indicated in the table. Each was incubated for the same length of time with the same amount of starch solution. From the results obtained we find that a 0.3% tannic acid solution in contact with diastase for any considerable time, in this case 18 hours, inhibits diastatic action.

In the experiments where tannic acid was used to precipitate proteins any excess beyond that which combined with the diastase (if such combination occurs) was removed almost immediately by filtration and washing.

7.3 Action Of Picric Acid Upon Diastase.
Experiment Number 8-4-21/2.

25. Cc. of a 50% aqueous acetone extract of malted wheat, as above described, were passed through a cellulose-aluminum phosphate prepared filter, which was then washed with distilled water until there was no test for sugar in the wash water as determined by Fehling's test.

The filter was now divided into two equal parts. One half of the filter was placed in a flask with 25. cc. of distilled water, and shaken until the filter was broken into small bits. The liquid was now incubated with an equal volume of a 1.% starch solution at 50.°C. A Fehling's test for sugar was made previous to incubation, with a negative result. After 3 hours incubation a Fehling's sugar determination was made which required 4.7 cc. of 0.1 n sodium thiosulphate solution. The blank test at this time was 0.5 cc. of 0.1 n thiosulphate.

The diastatic action is equivalent to 3.3 cc. of 0.1 n thiosulphate solution. The other half of the prepared filter which had not been treated, was placed in a flask containing 25. cc. of a saturated picric acid solution and shaken until finely divided and immediately wet with an equal volume of a 1.% starch solution. A sugar test by Fehling's method was then made with a negative result. The liquid was then incubated at 50.°C. for 3 hours and Fehling's sugar determination made which required 8.5 cc. of 0.1 n thiosulphate which is the same as the blank determination.

Table on Experiment Number C-4-23/2.

Composition of liquid	Sugar determination in cc. of 0.1 n sodium thiosulphate		
	Blank.	After 3 hours incubation.	Difference due to diastase
½ of filter paper and 25. cc. of H_2O. 25. cc. of 1.% starch solution.	0.5 cc.	4.7 cc.	3.3 cc.
½ of filter paper and 25. cc. of Sat. Picric Acid. 25. cc. 1.% starch solution.	0.5 cc.	8.5 cc.	0

In the above table here the diastase was extracted from one half of the filter with distilled water and incubated there for a

diastatic action equivalent to 5.0 cc. of thiosulphate. Pure concentrated picric acid was used as an extractant of diastase on the other half of the filter, and then incubated as the above, no diastatic action occured.

The conclusion from this experiment is that concentrated picric acid as used in this experiment inhibits diastatic action completely.

7.4 Action of H_2O_2 Upon Diastase.
Experiment Number 8-4-21/3.

10. cc. of a 50.% aqueous extract of malted wheat was passed through a cellulose-aluminum phosphate prepared filter as previously explained, and the adhering sugar removed in the usual way. The filter was now divided into two equal parts. One half was treated with 10. c . of H_2O_2, which had been neutralized with 2.5 cc. of a 0.02 n NaOH solution. This mixture was digested for 20 minutes and then it was incubated at 50.°C. for 1 hour and a Pang's sugar determination was made which required 5. cc. of hydroxylamine. The blank at this time on Pang's determination of sugar required 3.5 cc. of hydroxylamine to 5. cc. of copper solution.

The remaining half of the above filter was treated with 12.5 cc. of distilled H_2O and, after thoroughly shaking and standing for 20 minutes, was incubated 1 hour at 50°C. with an equal volume of a 1.% starch solution. A Pang's sugar determination was then made which required 2.8 cc. of hydroxylamine.

Table On Experiment Number 8-4-21/3.

	Sugar determinations in cc. of hydroxylamine.			
½ of filter 1..5 cc.H_2O reagent 7.5 cc.1. St.Sol.	Blank	After 1 hr. incubation.	Increase due to diastase.	Inhibition
	8.5 cc.	8.0 cc.	0.5 cc.	0.[illegible] cc.
½ of filter 12.5 cc.H_2O 12.5 cc.1. St. Sol.	8.5 cc.	7.8 cc.	0.7 cc.	

In the above it should be observed that diastase action took place where H_2O_2 was present as represented by 0.5 cc. of hydroxylamine. Where H_2O was the solvent we have the diastatic action equivalent to 0.7 cc. of hydroxylamine.

The conclusion is drawn that H_2O_2 as used in this experiment possibly inhibited diastatic action equivalent to 0.2 cc. of hydroxylamine, provided this small difference is greater than the limit of experimental error. H_2O_2 in the concentration used thus seems to interfere little if any with diastatic action.

7.5 Action Of Direct Sunlight Upon Diastase.
Experiment Number 8-4-16/1.

10. Cc. of a 50.% aqueous acetone extract of malted wheat was passed through a prepared filter and freed from the adhering sugar as previously described. The entire filter was placed in a flask with 25. cc. of distilled water and shaken until the filter was broken into small bits. A portion of the mixture was tested for diastase by the usual method of tests and incubation, with a positive result. Another portion of the water extract and filter was then placed in a test tube and tightly corked and placed in a south window exposed to the sunlight. After remaining in the window for 7 days, during which the sun shone about half

of the hour, this solution was incubated with an equal volume of a 1% starch solution and the usual tests were made and the solution was found to be still diastatically active.

8. Critical Estimation Of The Methods Developed And Summary Of Results.

As stated previously diastase is found in all known plant and animal tissue, and a number of methods have been presented for its extraction. The method of extraction depends quite largely upon the condition of the material which contains the diastase. Hence a number of conditions have to be considered in selecting the best method of extraction.

In extracting diastase from bacterial culture liquid some reagent must be added which will kill the bacteria and yet not interfere with the diastatic action. Toluol-thymol solution has been selected and found very satisfactory for our purpose. In fact it is indispensable in the methods of extracting diastase from bacterial culture liquids. It is used only as an antiseptic in the methods of extracting diastase from other compounds.

In the Acetone-Tannic acid method we find an excellent way of separating diastase from protein compounds such as are found in autolytic liver. This method is not found quite so advantageous when applied to carbohydrate material such as malted wheat.

The method of using the cellulose-aluminum-phosphate prepared filter was found very good, but much care and precaution is necessary in its application.

The principal objection to this method was some difficulty

found in centrifuging a liquid which contained aluminum phosphate and the time required for filtration.

In the preceding experiments where nine compounds were used the oxide and sulphate were found to inhibit diastatic action, and the zinc phosphate failed to give as good results as those obtained by lead phosphate as has previously been shown.

When efficiency, ease, and rapidity are considered the lead phosphate method is selected as being the best of the several methods which were tested.

Summary.

The work described in this paper may be briefly summarized as follows:-

1. The methods tried for the separation of diastase have been tested upon diastatic materials of different origin and contained in liquids of different compositions as follows:- Bacterial culture liquid, extract of autolytic liver, germinated wheat, etc.

2. A 50% aqueous acetone solution has been found to be a most efficient extracting agent for diastase. It gives a solution of the ferment nearly free from protein and carbohydrate colloids.

3. By extraction with a 50% aqueous acetone solution it has been found possible to extract the diastase from solid material thereby leaving the catalase wholly in the residue. With extract of autolytic liver the precipitating proteins, etc., carry down with them the catalase. The acetone medium does not inhibit the action of catalase when the latter is in solution.

4. Of the several methods investigated, all of which were more or less successful, the adsorption of diastase from aqueous or 50% acetone solution by normal lead phosphate, $Pb_3(PO_4)_2$, has been found efficient and expeditious. The adsorbed diastase is fully active in this condition. The lead phosphate also strongly accelerates the action of the diastase.

5. The diastase can be slowly dissolved from its adsorbent in active condition by distilled water.